U.S. Army Medical Research
Institute of Chemical Defense

USAMRICD-TR-12-05

Diet restriction and fasting exacerbate the toxicity of soman in young and old guinea pigs

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September 2012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
05-09-2012	Technical Report	01 March 2005-30 September 2005

4. TITLE AND SUBTITLE
Diet restriction and fasting exacerbate the toxicity of soman in young and old guinea pigs

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Myers, TM, Langston, JL

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-TR-12-05

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDZ
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)
MRICD

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

This research was supported by In-House Laboratory Independent Research (ILIR) funds awarded to T.M.M.

14. ABSTRACT

This study evaluated the effects of age (60 vs. 150 days), diet restriction (ad libitum vs. 80%), and fasting (recently fed vs. fasted 18 h) on survival, toxic signs, body weight, blood glucose, carboxylesterase, acetylcholinesterase, and butyrylcholinesterase in male guinea pigs exposed acutely to 0, 0.6, or 1.0 LD50 soman subcutaneously. Following soman exposure, body weight decreased but recovered by 1 week. Acetylcholinesterase levels were significantly decreased at all post-exposure time points (up to 1 week). Butyrylcholinesterase levels were suppressed out to 48 h post-exposure, but recovered by 1 week. Toxic signs were more severe in diet-restricted animals than ad libitum animals. Young animals exhibited more severe signs of toxicity than old animals. All animals in the saline and 0.6 LD50 groups survived to 1 week. For the 1.0 LD50 groups, old animals exhibited significantly greater survival (44.4%) than young animals (16.7%). Ad-libitum animals had significantly longer mean survival (87.7 h) times than diet-restricted animals (55.5 h), demonstrating a toxicity-enhancing effect of diet restriction.

15. SUBJECT TERMS
diet, caloric restriction, fasting, age, soman toxicity, acetylcholinesterase, body weight, guinea pig

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Todd M. Myers
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	Unlimited	20	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-8380

Abstract

This study evaluated the effects of age (60 vs. 150 days old), diet restriction (ad libitum feeding vs. 80% of the recommended amount), and fasting (fed 90 minutes prior vs. not fed for 18 h prior to exposure) on survival, toxic signs, body weight, blood glucose, carboxylesterase, acetylcholinesterase, and butyrylcholinesterase in male guinea pigs exposed acutely to 0, 0.6, or 1.0 LD_{50} soman subcutaneously. Just before soman exposure, body weights were lower in diet-restricted animals than in ad-lib fed animals, particularly for young guinea pigs. Glucose levels prior to soman challenge were higher in older guinea pigs compared to young guinea pigs and were higher in those under ad libitum feeding compared to those under diet restriction. Also at baseline, acetylcholinesterase and butyrylcholinesterase levels were higher in younger animals, but carboxylesterase levels were higher in older animals. Following soman exposure, body weight decreased but recovered by 1 week. Acetylcholinesterase levels were significantly decreased at all post-exposure time points (up to 1 week). Butyrylcholinesterase levels were suppressed out to 48 h post-exposure, but recovered by 1 week. Carboxylesterase and glucose levels did not exhibit dose-dependent changes following soman exposure. Diet-restricted animals had more severe signs of toxicity than ad libitum animals. Young animals exhibited more severe signs of toxicity than old animals. Toxic signs scores showed time-dependent changes and were highest at 1 h post-exposure, lower at 4 h, and even lower at 24 h. A dose-dependent effect was also observed in that the 1.0 LD_{50} group exhibited greater toxic signs than the 0.6 LD_{50} and saline groups. All animals in the saline and 0.6 LD_{50} groups survived to 1 week. For the 1.0 LD_{50} groups, old animals exhibited significantly greater survival (44.4%) than young animals (16.7%). *Ad-libitum* animals had significantly longer mean survival (87.7 h) times than *diet-restricted* animals (55.5 h), demonstrating a toxicity-enhancing effect of diet restriction. Old animals had significantly longer mean survival (95.6 h) times than young animals (47.6 h), demonstrating a toxicity-enhancing effect of age.

INTRODUCTION

Previous investigations of the effects of repeated sub-lethal exposure to chemical warfare nerve agents (CWNA) in guinea pigs have yielded conflicting results with respect to weight loss and the severity of other signs of toxicity (Hulet et al., 2002; Langston et al., 2005). Specifically, Hulet et al. (2002) reported no weight change in guinea pigs injected daily for two weeks with 0.4 LD_{50} sarin. In contrast, Langston et al. (2005) reported pronounced weight loss following the third consecutive daily injection of 0.4 LD_{50} sarin. There were several important methodological differences between these studies that may account for these differences: the guinea pigs in the Langston et al. (2005) study were older, under dietary regulation, and had been fasted for approximately 18 hours prior to exposure. The methodological differences were dictated primarily by the selection of behavioral assessments employed in the two studies: the Hulet et al. (2002) study examined changes in functional observational battery (FOB) performance, whereas Langston et al. (2005) examined changes in food-reinforced schedule-controlled operant behavior. The use of food-reinforced schedule-controlled operant behavior as a baseline for the detection of toxicant effects necessitates dietary regulation to ensure consistent levels of motivation, and animals are typically fed their daily food ration shortly after completing their training session. Furthermore, establishing stable baseline performance suitable for detecting toxicant effects can require several weeks to months of daily training sessions, resulting in substantially older animals at the time of toxicant exposure.

A limited number of studies with mice and rats have demonstrated increased CWNA toxicity in animals fasted for 18 hours prior to CWNA exposure (Clement et al., 1981; Clement, 1982; Fletcher et al., 1988). Clement and colleagues (1981, 1982) demonstrated the influence of an 18-h fast on the lethality of soman and HI-6 (1-[[[4(aminocarbonyl)-pyridinio]methoxy]methyl]-2(hydroxyimino)pyridinium dichloride) as well as the therapeutic efficacy of HI-6. There was a consistent trend toward increased lethality in fasted mice of various strains; however, this difference was only statistically significant for one strain. With respect to the oxime HI-6, fasting increased both its lethality and its therapeutic efficacy. Fletcher et al. (1988) demonstrated that fasted rats had more severe signs of toxicity at 30 min following soman exposure. Fasted rats also had reduced blood glucose and insulin levels; however, fasting increased blood glucagon, corticosterone, and norepinephrine levels.

In addition to fasting, *long-term* dietary restriction may also have effects on CWNA toxicity. It is well established that long-term dietary restriction increases longevity and reduces the incidence of disease in a variety of species (Keenan et al., 1997; Roth et al., 2000), but the effects of this variable on toxicity are poorly understood. A limited number of studies suggest that the toxicity produced by common pharmaceutical compounds may actually be *decreased* by long-term dietary restriction (Keenan et al., 1996). There have been no published reports that have evaluated this issue; however, it may be an important predictor of human health effects in a CWNA-exposure scenario, with individuals of different body composition exhibiting different sensitivities to CWNA.

The effects of age on the toxicity of a number of agents, including organophosphorus compounds, have been studied more extensively, but the majority of these studies has focused on the very young and, thus, may not be relevant to the military's interest in this factor. For example, Moser et al. (1998) evaluated groups of rats 17, 27, and 70 days old representing preweanling, postweanling, and young adult, respectively. In general, pre- and postweanling rats were more sensitive than young adult rats to chlorpyrifos intoxication, presumably due to lower esterase levels in the very young. With respect to CWNA, one study evaluated the effects of age

in a range more relevant to the (adult) military population and demonstrated convincingly that older rats exhibit greater CWNA toxicity (Shih et al., 1990). Shih and colleagues studied four groups of rats (fed ad libitum), 30, 60, 120, and 240 days old. The calculated 24-h LD_{50}s were 110, 87, 66 and 59 micrograms/kg, IM, for 30-, 60-, 120- and 240-day-old rats, respectively. A significant and positive age-related effect on toxic sign rating scores was observed at one hour following soman injection. Thus, for both LD_{50} and toxic signs, a direct linear relation was observed between age and soman toxicity. A separate study in mice corroborated these basic findings, demonstrating that the LD_{50} of soman peaked at 30 days of age and was nearly 40% lower by 120 days of age and thereafter (Peet et al., 1987). Thus, in both rats and mice, soman toxicity appears to increase with age.

The guinea pig is commonly viewed as the rodent species of choice for CWNA research because it has lower endogenous levels of plasma carboxylesterase (Benschop and De Jong, 1991; Fonnum and Sterri, 1981; Inns and Leadbeater, 1983; Sterri et al., 1980; Sterri et al., 1981). Carboxylesterase levels appear to be responsible for species differences in sensitivity to CWNA toxicity (Maxwell et al., 1987). Since carboxylesterase can function as an endogenous CWNA scavenger (Maxwell et al., 1987), the lower carboxylesterase levels found in guinea pigs result in toxicological responses to a given dose of CWNA that are more similar to those of non-human primates than to those of rats and mice. Additionally, therapeutics often show greater efficacy in guinea pigs than in other rodent species (de Groot et al., 2001; Gordon et al., 1978). Furthermore, the toxicokinetic profile of organophosphorus compounds in guinea pigs is considered to be more similar to that in marmoset monkeys than is that of the rat (Benschop and De Jong, 1991).

Similar to the Shih et al. (1990) study outlined above, the impetus for this study grew out of our observations (published and unpublished) from a number of different studies examining the behavioral toxicity of repeated CWNA exposure in guinea pigs. Across those studies, we routinely observed enhanced toxicity in animals that had an extensive history of behavioral training, compared to historical data, and suspected that age and fasting may be responsible for those results. Furthermore, we speculated that dietary restriction may be having additional effects on the toxic response. The major goal of the present research was to replicate and extend to guinea pigs the findings previously reported with rats and mice. In particular, the effects of age on CWNA toxicity may have a direct impact on the interpretation of studies using neurobehavioral assessments in guinea pigs, given the extensive time necessary to train the animals. Additionally, because those studies utilized food-reinforced procedures, both short-term and long-term dietary restrictions were used to varying degrees. In addition to directly addressing these methodological concerns, this research is the first to simultaneously evaluate the effects of age, fasting, and long-term dietary restriction on CWNA toxicity.

METHOD

Subjects

Male Hartley (Crl:HA) guinea pigs (*Cavia porcellus*) were obtained from Charles River Laboratories Canada at two different ages, 3 weeks and 6 weeks. Animals were housed individually in polycarbonate cages with free access to tap water provided by bottle. The animal colony was maintained in fully AAALAC-accredited facilities on a 12-h light/dark cycle with lights on at 0600 h and at 20-22°C with a relative humidity of 50% (±15%), using at least 10 complete air changes per hour of 100% conditioned fresh air. All animals had ad libitum access to food within 30 minutes following exposure and for the remainder of the study.

Procedure

Animals within each age group were randomly divided into two long-term diet groups (ad libitum [AL] versus 80% of the recommended daily amount [DR]) and maintained on that diet regimen for at least four weeks prior to the evaluation of soman toxicity. Prior to soman exposure, these four groups of animals were further subdivided into eight equal groups, those fasted (F) 18 hours prior to soman exposure versus those fed (PO) 60-90 minutes prior to soman exposure. Table 1 summarizes the three independent variables and the eight different experimental groups, with 21 total animals in each group.

Table 1

Long-Term Diet	Short-Term Diet	Age	Soman Dose	N
Ad-libitum (AL)	Fed (PO)	60 (Young)	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9
		150 (Old)	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9
	Fasted (F)	60	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9
		150	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9
Restricted (DR)	Fed (PO)	60	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9
		150	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9
	Fasted (F)	60	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9
		150	Saline	3
			0.6 LD_{50}	9
			1.0 LD_{50}	9

Dietary restriction consisted of feeding the animals a weighed amount of guinea pig chow each afternoon according to nutritional guidelines set forth by Charles River and the Institute for Laboratory Animal Research (Subcommittee on Laboratory Animal Nutrition, 1995). This amount is 80% of the amount typically consumed by healthy male guinea pigs. The amount allotted to each individual guinea pig equaled 0.80 X 60 g/kg body weight per day (c.f. Kind et al., 1999; Kind et al., 2002; Olausson and Sohlstrom, 2003; Sohlstrom et al., 1998). For fasted animals, food was removed 18 h prior to exposure. For PO animals, food was presented 60-90 minutes prior to exposure (in the case of ad lib animals, this feeding manipulation resulted in no change in food access).

Soman exposure

Soman (GD, pinacolyl methylphosphonofluoridate; US Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA) toxicity was assessed using three different doses within each of eight groups: saline control (n=3), 0.6 LD_{50} (n=9), and 1.0 LD_{50} (n=9). Exposures occurred at approximately 0930. Saline or soman was injected subcutaneously in the right flank. All injections were performed using 1-ml syringes with 25-gauge, 3/8-inch needles. Soman was prepared at two different concentrations (33.6 μg/ml to deliver 16.8 μg/kg or 0.6 LD_{50}; 56 μg/ml to deliver 28 μg/kg or 1.0 LD_{50}), keeping all injection volumes constant at 0.5 ml/kg. An equivalent volume of saline was injected into control animals. The subcutaneous LD_{50} of soman in ad libitum, unfasted, 35-day-old guinea pigs is 28 μg/kg (Capacio et al., 2004; Maxwell et al., 1988; McDonough et al., 2004). Survival was recorded at 1 h, 24 h, 48 h, and 168 h (1 week). Animals that survived 1 week post-exposure were anesthetized with pentobarbital and perfused with saline and formalin to allow for histochemical evaluation of brain injury.

Biochemical analyses

For all guinea pigs, blood samples were obtained via toenail clip (Vallejo-Freire, 1951) prior to soman or saline administration (at minus 1 week and at 0 h) and analyzed for acetylcholinesterase, butyrylcholinesterase, carboxylesterase, and glucose. In survivors, post-exposure samples were taken at 4 h, 24 h, 48 h, and 1 week post-exposure. Blood samples (0.3 to 1.0 ml) were collected into EDTA-prepared microtubes and submitted for glucose analysis (Nova Biomedical pHOx Plus STAT) and esterase analyses using the WRAIR method for acetylcholinesterase and butyrylcholinesterase and the titrimetric method with tributyrin as substrate for carboxylesterase assay. The 1-week blood sample was collected via heart stick following anesthetization with 75 mg/kg sodium pentobarbital injected intraperitoneally (i.p.).

Neuropathological assessment

Animals that survived 1 week were deeply anesthetized with sodium pentobarbital (75.0 mg/kg, i.p.) and euthanized by exsanguination via perfusion through the aorta with saline, followed by 10% phosphate buffered formalin (PBF). Following post-fixation in PBF for at least 24 h at 4°C, brains were coronally cut into 3-mm slices. Brain slices at the level of the dorsal hippocampus were paraffin-processed and sectioned at 5–10 μm. Sections between bregma −2.52 and −3.12 mm were stained with hematoxylin and eosin and evaluated by a pathologist who was unaware of the experimental history of a given subject. These coordinates contain brain regions that are known to be susceptible to nerve agent-induced damage (McDonough, Jr. et al., 2000). In the present study, six brain regions (piriform cortex, amygdala, hippocampus, thalamus, dorsal cortices, and lateral cortices) were qualitatively scored on a scale from 0–4: 0 =

no damage, 1 = minimal damage (1–10% necrotic neurons), 2 = mild (11–25%), 3 = moderate (26–45%), and 4 = severe (> 45%) (McDonough, Jr. et al., 1995; McDonough, Jr. et al., 2000; Shih et al., 2003). The magnitude of total brain damage was assessed by summing the neuropathology scores of the six areas.

Signs of Toxicity

Toxic signs and body weights were evaluated at 0 h, 1 h, 4 h, 24 h, and 1 week post-exposure according to previously established methods (c.f., Jovic, 1974; McDonough, Jr. et al., 1986). Signs scored as present or absent included mastication, salivation, lacrimation, and piloerection. Signs scored on a 4-point level of severity included general motor signs (e.g, tremor) and locomotor activity (e.g., impaired movement). Each sign was scored separately, and all were added to compute an overall toxicity score for each guinea pig at each time point for purposes of data analysis. On most occasions, a second observer independently scored the toxic signs so that interobserver agreement scores could be calculated.

Data Analysis

Visual analysis and descriptive statistics were initially used to evaluate all data. The design is a 2 (age; 60 [young] versus 150 days [old]) x 2 (long-term diet; ad libitum [AL] versus 80% of the recommended amount [DR]) x 2 (fasting; fasted 18 h [F] versus fed 1 h prior [PO]) x 3 (dose; saline, 0.6 LD_{50}, or 1.0 LD_{50} soman) for a total of 24 treatment groups (i.e., cells) in this factorial repeated-measures design. Percent survival at 1 h, 4 h, 24 h, 48 h, and 1 week was of primary interest. Survival analyses were conducted using S-PLUS® 7.0 software (Insightful Corporation, Seattle, WA, USA) using the *survfit* function, and differences between groups at each time point were evaluated using the *survdiff* function. Adjustments to the critical *p* values for multiple comparisons were made using the Sidak correction procedure. This approach fits a Kaplan-Meier survival curve and compares groups based on the Fleming-Harrington Gρ family of tests (S-PLUS 7 Guide to Statistics, Volume 2, Insightful Corporation, Seattle, WA, USA, 2005). Also of interest were acetylcholinesterase, butyrylcholinesterase, carboxylesterase, and glucose levels, body weight, and toxic signs at each time point and across time. Linear mixed effects models (S-PLUS *lme* function) were fitted to the biochemical, toxic sign, and body weight data. For these models, age, long-term diet, fasting, dose and time were fixed effects, and subject was treated as a random effect. A significance level of $p < .05$ was used for all tests.

RESULTS

Survival

Survival was assessed at 1 h, 4 h, 24 h, 48 h, and 1week after exposure. All animals in the saline and 0.6 LD_{50} groups survived to 1 week (data not shown). Thus, for the subsequent analyses, only the data from the 1.0 LD_{50} groups were used. Among the young animals in the AL-PO group, survival at 24 h and 1 week approximated the LD_{50} (56% and 44%, respectively). In contrast, no young animals in the DR-F group survived longer than 24 h. By 24 h, survival in the young DR-PO group was 11% and remained unchanged at 1 week. Survival in the young AL-F group equaled 33% at 24 h but by the end of 1 week had decreased to 11%. Among the old animals, survival at 24 h in the AL-PO group approximated the LD_{50} at 56% and remained unchanged at 1 week. The old DR-PO group had 67% survival at 24 h, but by 1 week that had decreased to 56%. Among the old AL-F animals, survival at 24 h was 67% and at 1 week was 44%. The old DR-F animals fared much worse with only 22% survival at 24 h; however, this

remained stable by the end of 1 week. The main effect of age was statistically significant in that old animals exhibited greater [$\chi^2(1) = 9.5$, $p < .003$] survival (44%) than young animals (17%). Similarly, the simple main effect of age in DR animals (collapsed across fasted/fed) revealed that young DR animals exhibited significantly [$\chi^2(1) = 9.3$, $p < .003$] less survival (6%) than the old DR (39%) animals. Mean survival times were also calculated for each group. DR animals had significantly shorter survival times compared to AL animals [$F(1, 64) = 4.387$, $p = .04$]. Figure 1 shows the proportion surviving for the young and old animals in the various diet groups after receiving a 1.0 LD_{50} dose of soman.

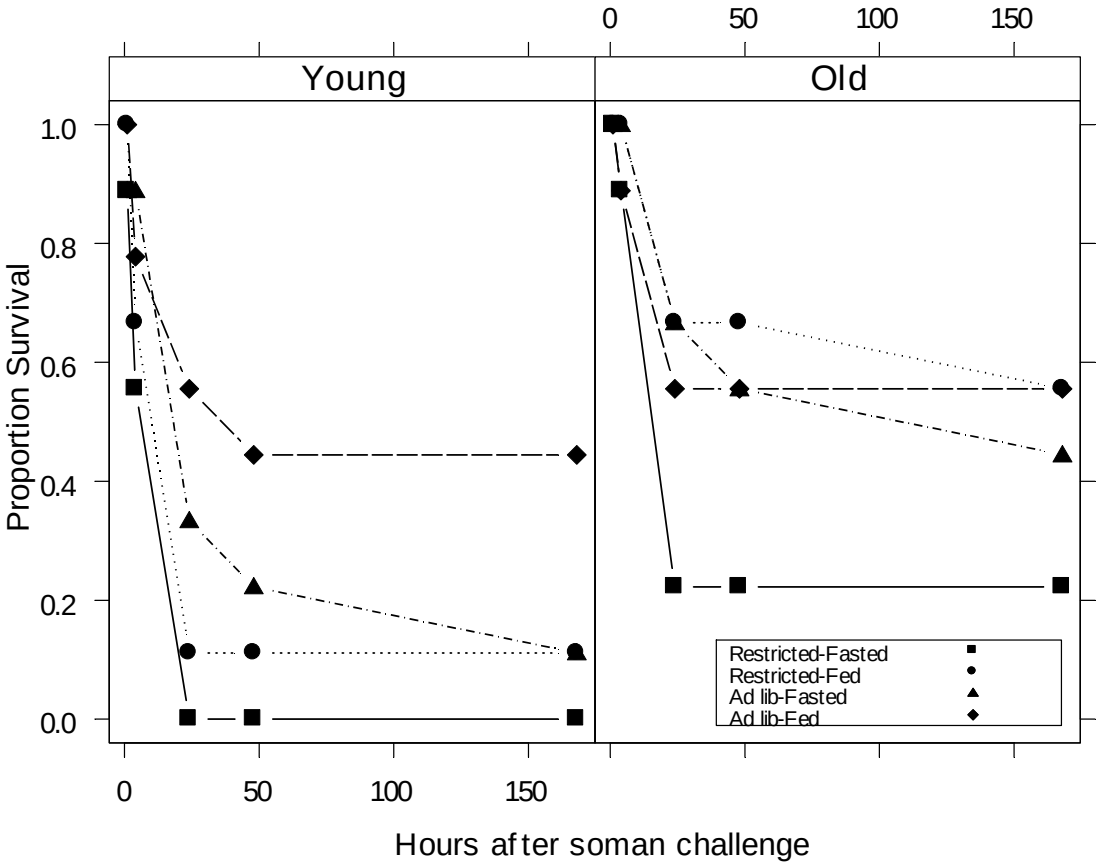

FIGURE 1. Proportion survival following soman (28 μg/kg) as a function of age, long-term diet, and fasting.

Histopathology

Histopathology assessments indicated a detectable neuropathology in approximately 24% of the animals surviving 1 week following exposure to 1.0 LD_{50} soman. There was no incidence of neuropathology in control animals (0/24) or animals exposed to 0.6 LD_{50} soman (0/66). There was a higher prevalence of neuropathology in young animals (60%; 3 of 5 animals) than in old animals (12.5%; 2 of 16 animals). Among young animals, one had diffuse mild pathology, and the other two had mild or severe localized (lateral cortices: parietal/temporal) pathology. Among the old animals, the two had minimal or moderate pathology localized in the lateral cortices.

Toxic Signs

Toxic sign scores were taken at 1, 4, and 24 h after exposure. Interobserver agreement scores were calculated by correlating the scores of Observer 1 with those of Observer 2 at each time point and computing r^2. Interobserver agreement scores were high, ranging from 0.86 to 0.93 and averaging 0.89 for both experiments (young and old) at all three time points (1, 4, and 24 h). Overall, the toxic signs scores were greater in animals administered 1.0 LD_{50} soman than in animals administered either saline or 0.6 LD_{50} soman [$F(2, 138) = 29.7$, $p < .0001$]. Toxic signs scores at 1 h were significantly higher than those at 4 h which were significantly higher than those at 24 h [$F(2, 236) = 41$, $p < .0001$]. Among animals exposed to 1.0 LD_{50} soman young DR animals had greater toxic signs scores than old DR animals. Among young animals exposed to 1.0 LD_{50} soman, toxic signs scores of DR animals were greater than those of AL animals. The upper panels of Figure 2 show the results for the young animals as a function of dose and diet restriction level and the lower panels show similar results for the old animals.

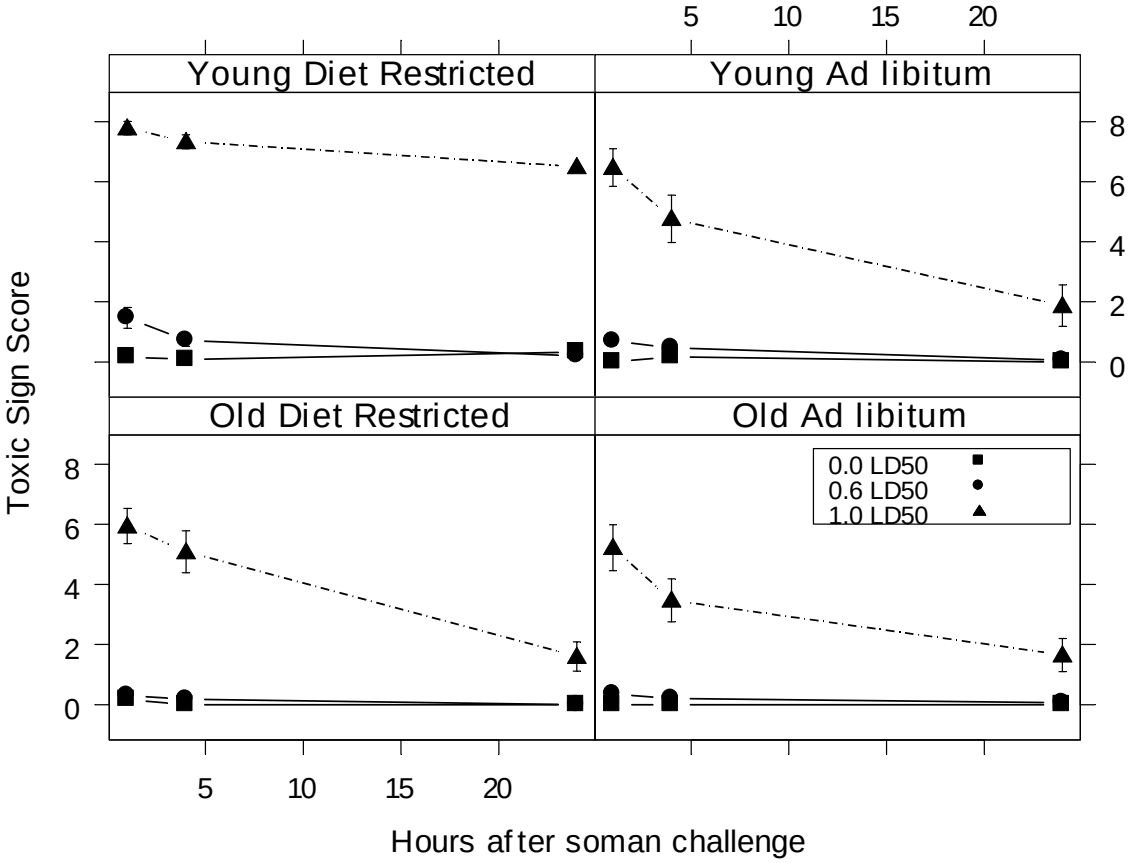

FIGURE 2. Toxic signs as a function of age, long-term diet, and soman dose.

Body Weight

There were no significant changes in body weight for animals administered saline or 0.6 LD_{50} soman (data not shown or discussed further). Changes in body weight following exposure to 1.0 LD_{50} soman are shown in Figure 3. Within the young animals, the AL group weighed more than DR group [$F(1, 141) = 22$, $p < .0001$]. Additionally, body weight was significantly

higher at 0 h than at 4 h and 24 h; the 48-h weight was significantly greater than the 4- and 24-h weights, and the 1-week weight was significantly greater than all others. For old animals, there were no significant differences as a function of either fasting or long-term diet. The 0-h weight was significantly greater than the 4-, 24-, and 48-h weights, and the 1-week weight was significantly greater than the 4-, 24-, and 48-h weights.

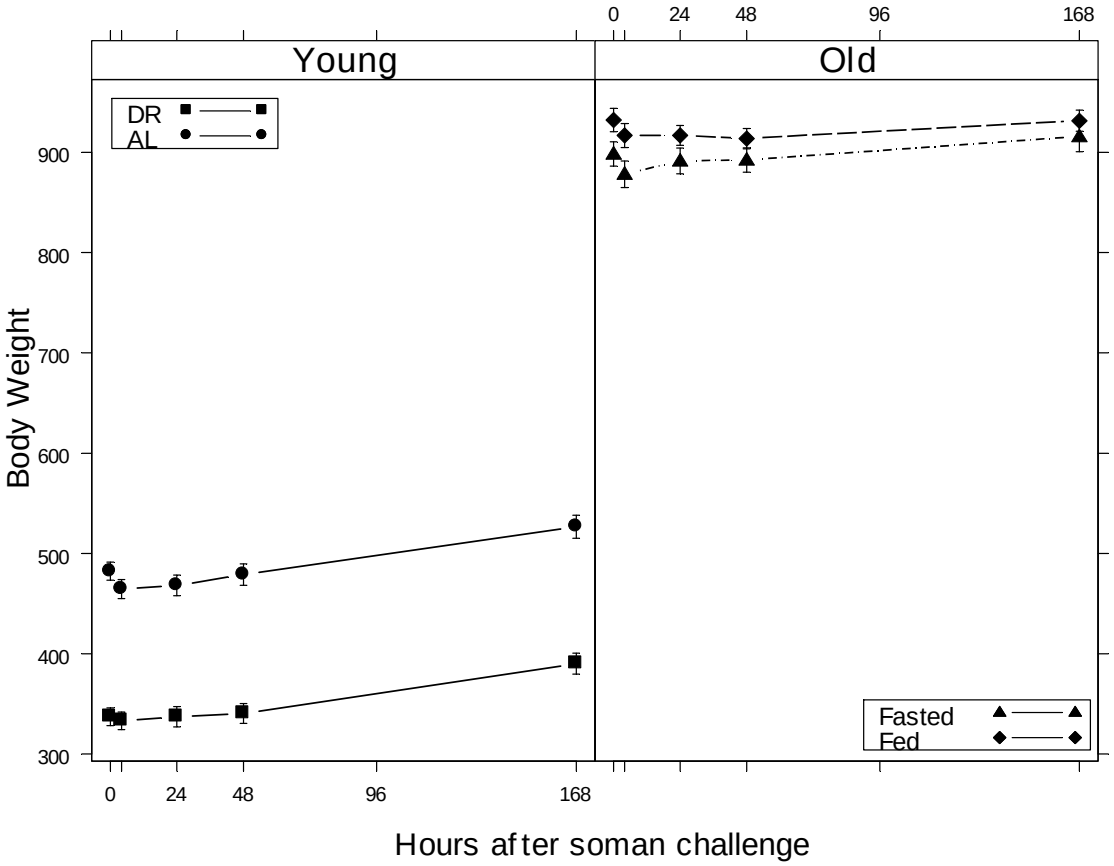

FIGURE 3. Post-exposure body weights for young and old guinea pigs as a function of long-term or short-term diet restriction, respectively.

Acetylcholinesterase

Acetylcholinesterase (AChE) was measured 1 week prior to exposure (baseline), minutes prior to exposure (0 h) and at 4 h, 24 h, 48 h, and 1 week after exposure. At baseline, the AL group had significantly greater AChE activity than DR group [$F(1, 590) = 10$, $p < .002$; data not shown]. Also at baseline, young animals had greater AChE activity than old animals [$F(1, 590) = 6$, $p < .02$; data not shown]. At post-exposure time points of 4 h and greater, the 0.6 and 1.0 LD_{50} groups had significantly less AChE activity than the saline control group [$F(2, 590) = 21$, $p < .0001$]. Figure 4 shows the time course of AChE activity in each dose group for each age group (young in the left panel and old in the right panel). The dose-time functions were similar for both age groups. Within the young saline group, the 0-h AChE activity was equivalent to that at all other time points. In the old saline group, the 1-week AChE activity was significantly greater

than all previous time points. In contrast, for the 0.6 and 1.0 LD_{50} groups, the 0-h AChE activity was significantly greater than AChE activity at all subsequent time points.

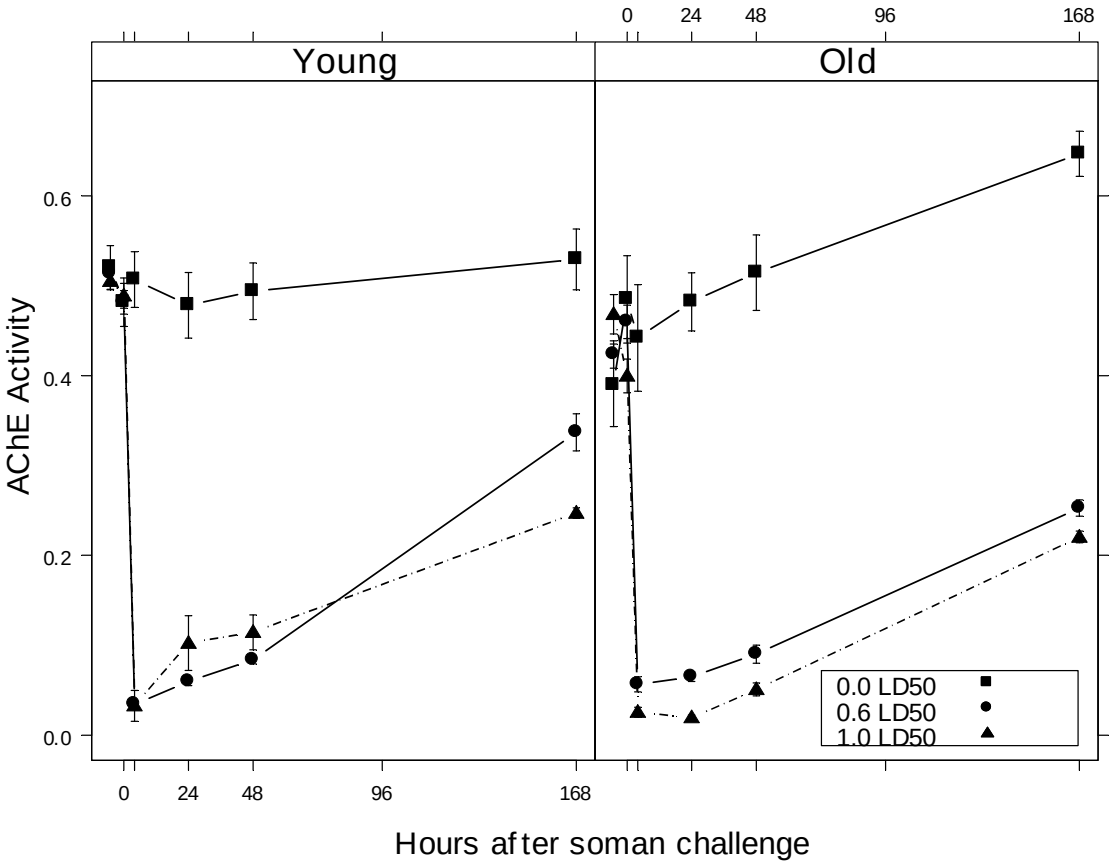

FIGURE 4. Post-exposure acetylcholinesterase levels as a function of age and soman dose.

Butyrylcholinesterase

Butyrylcholinesterase (BChE) was measured 1 week prior to exposure (baseline), minutes prior to exposure (0 h) and at 4 h, 24 h, 48 h, and 1 week after exposure. The main effect of age was significant in that young animals had significantly higher BChE activity than older animals [$F(1, 141) = 11$, $p < .002$]. The main effect of dose was significant and revealed that the saline control group exhibited higher BChE activity than both soman-exposed groups [$F(2, 141) = 9$, $p < .001$]. Among the soman groups, the 0.6 LD_{50} group had greater BChE activity than the 1.0 LD_{50} group. Overall, the 0 h and 1 week time points showed greater BChE activity than all other time points (4, 24, and 48 h). BChE activity at 48 h was greater than at 4 and 24 h. BChE activity at 24 h was significantly greater than at 4 h. Within the saline control group, BChE activity was similar at all time points. In contrast, for the 0.6 and 1.0 LD_{50} groups, BChE activity at the 0 and 168 h time points was greater than at 4, 24, and 48 h. Overall, BChE activity was greater in the younger animals than in the older animals. See Figure 5.

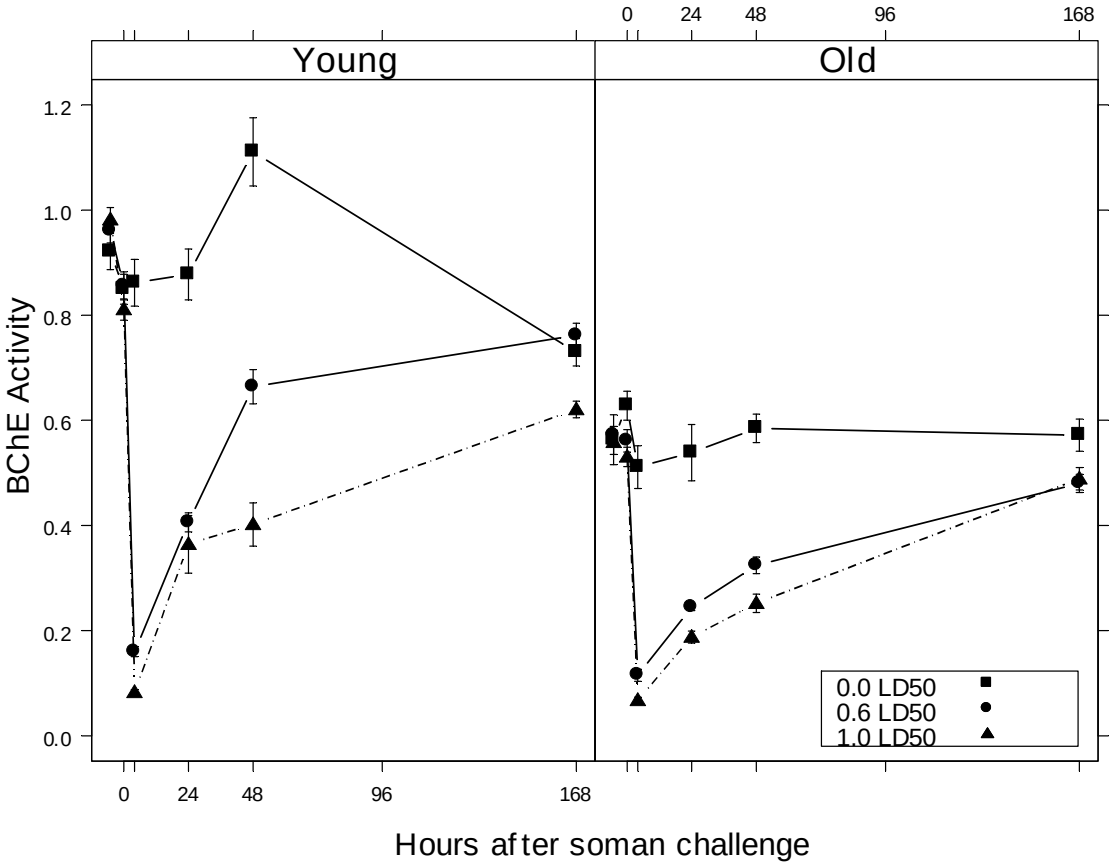

FIGURE 5. Post-exposure butyrylcholinesterase levels as a function of age and soman dose.

Carboxylesterase

Carboxylesterase (CaE) was measured 1 week prior to exposure (baseline), minutes prior to exposure (0 h) and at 4 h, 24 h, 48 h, and 1 week after exposure. At baseline, older animals had significantly greater CaE activity than young animals [$F(2, 631) = 5$, $p < .01$]. Figure 6 shows the results for CaE across time for young and old guinea pigs. Overall, the older animals exhibited significantly greater CaE activity than the young animals during the post-exposure period [$F(6, 631) = 3$, $p < .05$]. Overall, CaE activity was greater at 1 week post-exposure than at 4, 24, and 48 h post-exposure. No effect of dose was detected, and this was likely a result of the high degree of both inter- and intrasubject variability in CaE activity measurements.

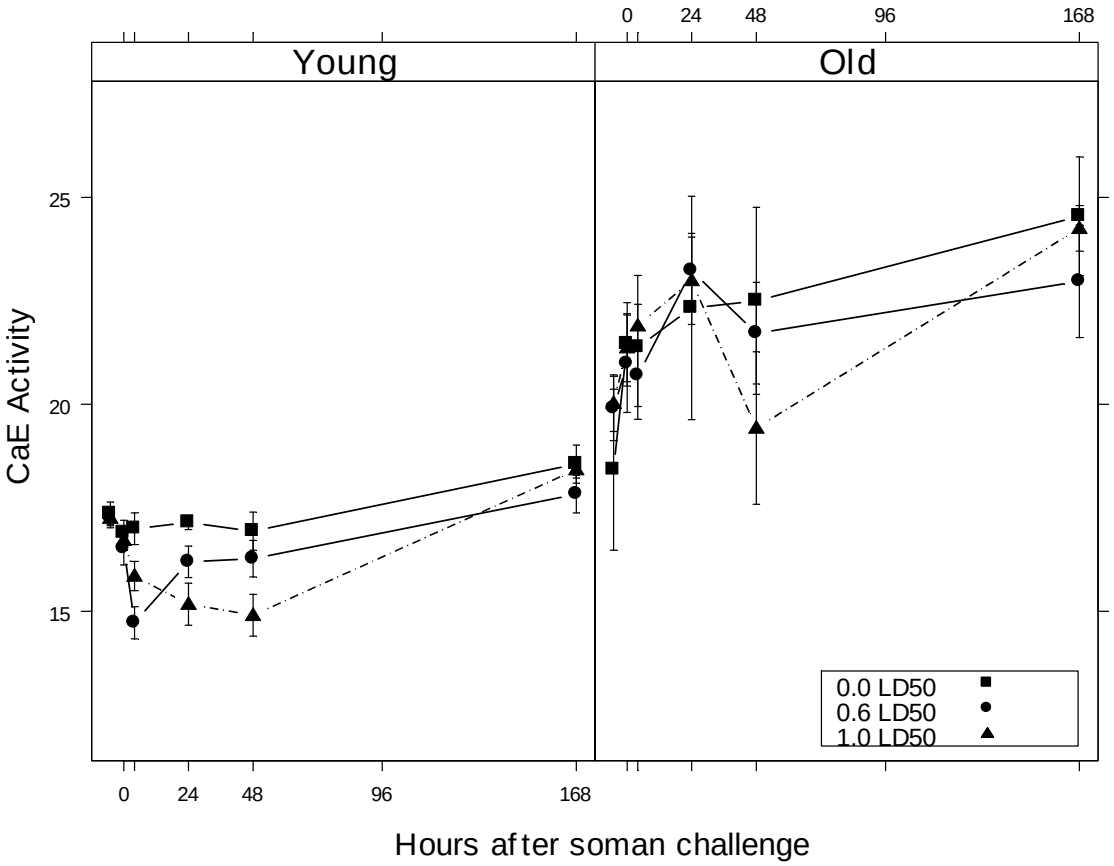

FIGURE 6. Post-exposure carboxylesterase levels as a function of age and soman dose.

Glucose

Glucose was measured 1 week prior to exposure (baseline), minutes prior to exposure (0 h) and at 4 h, 24 h, 48 h, and 1 week after exposure. At baseline, older animals had significantly greater glucose levels than younger animals [$F(2, 585) = 20.1$, $p < .0001$]. Also at baseline, the AL group had significantly greater blood glucose levels than the DR group [$F(2, 585) = 23.2$, $p < .0001$; data not shown]. Glucose levels at 48 h were greater than those at the baseline, 0, and 4-h time points. Overall, the glucose level was significantly greater for the PO groups relative to F groups [$F(1, 141) = 3.9$, $p = .05$; data not shown]. Figure 7 shows these results.

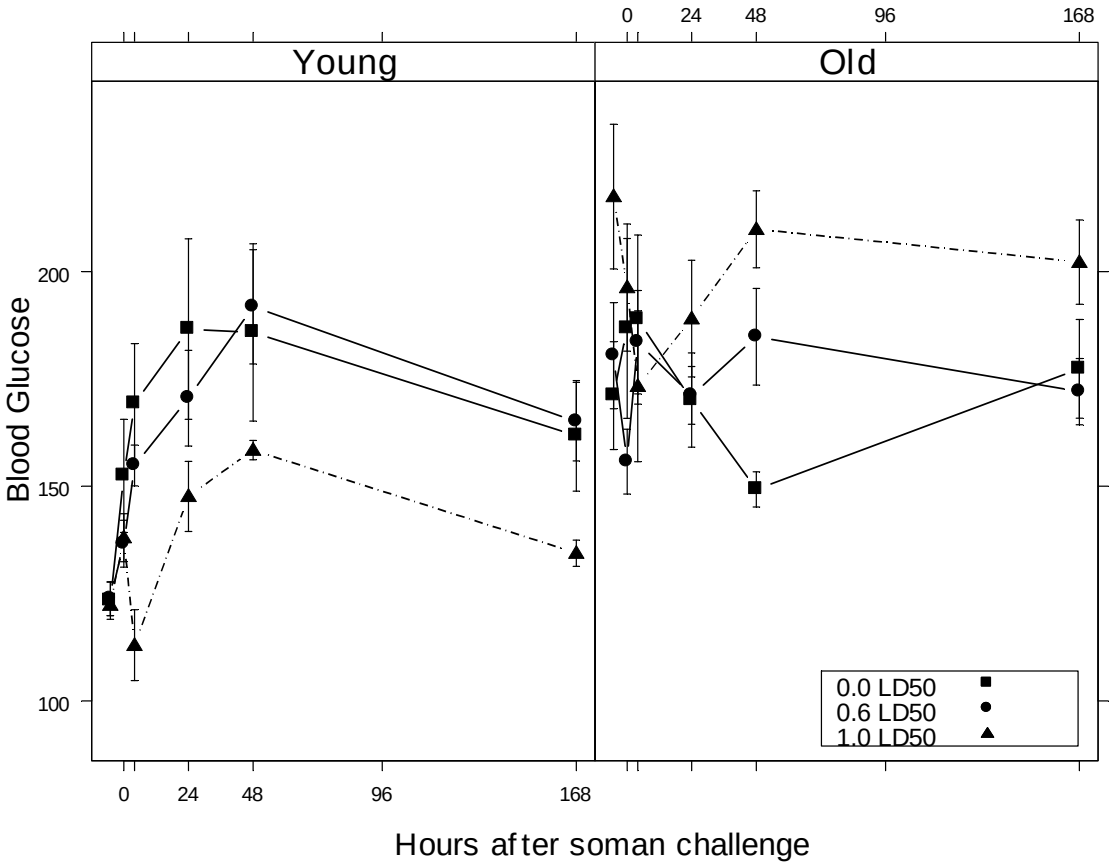

FIGURE 7. Post-exposure blood glucose levels as a function of age and soman dose.

DISCUSSION

The present data indicate that young animals that were fasted and/or under DR were more susceptible to the toxic effects of a nominal 1.0 LD_{50} dose of soman than were AL animals with access to food immediately prior to soman. Similarly, among older animals, those under DR and fasted prior to exposure had increased sensitivity to the toxic effects of a nominal 1.0 LD_{50} dose of soman. Signs of soman toxicity were more pronounced in DR groups than in AL groups. Young animals, likewise, had more pronounced signs of soman toxicity than older animals. The effects of DR were more potent in the young animals than in the old animals. There were greater differences in body weight, as a function of long-term diet, among young animals (26%) than among old animals (4.8%). Following soman exposure, weight loss occurred in both young and old animals. Body weights had recovered by 48 h and 1 week in the young and old groups, respectively. By 1 week, the young animals had surpassed their 0-h weight. This was not the case in the older animals, underscoring the different growth rates of these two different age groups.

With respect to enzymatic activity, at baseline the AL groups had higher AChE activity than the DR groups, and young animals had higher AChE activity than older animals. Furthermore, at baseline, young animals also had significantly greater BChE activity than older animals. Young DR animals had significantly less BChE activity than young AL animals. Conversely, old DR animals had significantly greater BChE activity than old AL animals. Older animals had significantly greater CaE activity than younger animals at baseline. Glucose levels were higher in older animals than younger animals, and AL groups had significantly higher glucose levels than DR groups.

The effects of age on nerve agent toxicity have been studied to a limited extent. Shih and colleagues (1990) studied four groups of rats (all fed ad libitum), 30, 60, 120, and 240 days old. Using an up-down method, the calculated 24-h LD_{50}s were 110, 87, 66 and 59 μg/kg, IM, for 30, 60, 120 and 240 day old rats, respectively. A significant and positive age-related effect on toxic sign rating scores was observed at one hour following soman injection. Thus, for both LD_{50} and toxic signs, a direct linear relation was observed between age and soman toxicity. Peet et al. (1987) corroborated these basic findings in mice (also fed ad libitum). The LD_{50} of soman peaked at 30 days old (130 μg /kg SC). By 90 days old, the LD_{50} was significantly lower (110 μg /kg). By 120 days old and thereafter, the LD_{50} stabilized at approximately 85 μg /kg. A strong (but imperfect) correlation between the LD_{50} and serum CaE levels was observed, suggesting that serum CaE is an important detoxifying route following subcutaneous soman exposure in mice. Based on these studies, soman toxicity appears to increase with age in both mice and rats. A recent report indicated that the toxicity of sarin and VX increased with age in male guinea pigs; however, the toxicity of soman did not change as a function of age (Fawcett et al., 2009). The present findings with AL-PO animals are in agreement with these findings.

In general, there appears to be a positive relationship between age and CWNA toxicity; however, some unresolved discrepancies and inconsistencies are present in the data for guinea pigs. One factor that should be investigated is the developmental time-course of enzymes in guinea pigs relative to rats and mice. In the Peet et al. (1987) study cited above, the serum CaE level increased sharply across the first 45 days of life. Similarly, it is well documented that the AChE level of very young (preweanling) rats is much lower than that of young adult rats (e.g., Moser et al., 1998). Very young rodents exhibit increased toxicity to soman which is highly and positively correlated with the development of plasma CaE levels (Fonnum et al., 1985). Although studies of esterase development in guinea pigs are lacking, the longer life span of guinea pigs (approximately 5 years) relative to mice and rats (approximately 2 years) suggests

that the esterase levels in the young guinea pigs used in the present study may not have reached maximal levels. Indeed, the older (150-day-old) animals had significantly higher levels of CaE than did young animals, and this may have been responsible for the reduced toxic signs and mortality observed in the older animals. However, for both AChE and BChE, *younger* animals had significantly higher levels (although the absolute difference was rather small for AChE). Post-exposure changes in AChE and BChE were similar for young and old animals.

Fletcher et al. (1988) demonstrated that fasting increased toxic sign scores in rats following exposure to soman (80 μg/kg, SC). Clement et al. (1981) demonstrated that, in only one of eight mouse strains tested, fasting significantly decreased the LD_{50} of soman. In the present study, fasting was generally without effect on toxic signs and survival, except that old-DR animals had longer mean survival times when they were fed rather than fasted prior to soman challenge. It is noteworthy that, for young guinea pigs, only one of eighteen fasted animals survived. However, because of significant mortality in the PO groups, statistical significance was not achieved. It is possible that increasing the sample size would allow a significant effect of fasting on survival to be shown.

Long-term dietary restriction increases longevity and reduces the incidence of disease in a variety of species (Keenan et al., 1997; Roth et al., 2000), but the effects of this variable on toxicity are not well understood. Keenan et al. (1996) reported that some common pharmaceutical compounds (not organophosphorus compounds) may be less toxic in long-term diet-restricted animals. In the present study, DR decreased AChE overall. DR decreased BChE in young animals but increased BChE in older animals. DR increased toxic sign scores overall. Among old-F animals, those that were DR exhibited a decreased mean survival time. These last two findings indicate that DR may enhance the toxicity of soman.

In summary, the present results indicate that age and dietary variables play important roles in the toxicity of CWNAs in guinea pigs. Considering toxic signs and survival together, the combination of both fasting and long-term DR appeared to increase soman toxicity in both age groups (DR-F animals of both ages). Overall, young animals showed greater toxicity to soman than old animals. The notable exception was old DR-F animals. Guinea pigs of similar age and dietary status are commonly employed in operant testing (Langston et al., 2005), motivated by long-term DR and overnight fasting. Considerable training (e.g., 6-36 weeks of daily testing) necessary to produce complex behavioral performances results in animals that are older at the time of nerve agent exposure. The present results indicate that animals such as these will have enhanced toxic responses to "reference" doses (i.e., those obtained with young AL-PO animals) of CWNA. The relative importance of the factors, based on the present study, might suggest age to be the most important factor in this subset of research animals. However, this is primarily due to the toxicity-enhancing effects of DR and fasting in young animals. When using young animals, it is recommended that behavioral tests employ non-appetitive reinforcers (aversive stimuli) to avoid confounding assessments of neurobehavioral toxicity with overt toxicity. Alternatively, verifying the LD_{50} in a subset of animals with the same age, dietary restriction, and fasting status for an individual study prior to the full-scale dose-response behavioral toxicity assessment will ensure appropriate dosing "to effect."

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